Description

Recombinant Microorganism

Technical Field

The present invention relates to a recombinant microorganism which may be used to produce useful proteins or polypeptides, as well as to such proteins and polypeptides.

Technical Background

Microorganisms are widely used for industrially producing a broad range of useful substances, including alcoholic beverages, certain types of foods such as *miso* and *shoyu*, amino acids, organic acids, nucleic-acid-related substances, antibiotics, sugars, lipids, and proteins. These substances also find diversified uses, including foods, pharmaceuticals, detergents, products for daily use such as cosmetics, and a variety of chemical raw materials.

In industrial production of useful substances by use of microorganisms, improvement of productivity is one major topic of interest, and one approach therefor is breeding of microorganisms through mutagenesis or other genetic means. Recently, in particular, with advancement of microbial genetics and biotechnology, more efficient breeding of useful microorganisms is performed through gene recombination techniques, and in association therewith, host microorganisms for obtaining recombinant genes are under development. For

example, *Bacillus subtilis* Marburg No. 168, which has already been confirmed to be safe and have excellent characteristics as a host microorganism, has been further improved.

However, microorganisms inherently possess diversified genes so that they can cope with environmental changes in the natural world, and thus, they do not necessarily exhibit high production efficiency of proteins or similar substances in industrial production, where only limited production media are employed.

Disclosure of the Invention

The present invention provides a recombinant microorganism prepared by transferring, to a mutant strain of microorganism from which any of *Bacillus subtilis* genes *comA*, *yopO*, *treR*, *yvbA*, *cspB*, *yvaN*, *yttP*, *yurK*, *yozA*, *licR*, *sigL*, *mntR*, *glcT*, *yvdE*, *ykvE*, *slr*, *rocR*, *ccpA*, *yaaT*, *yyaA*, *yycH*, *yacP*, *hprK*, *rsiX*, *yhdK*, and *ylbO*, or one or more genes functionally equivalent to any of these genes have been deleted or knocked out, a gene encoding a heterologous protein or polypeptide.

Brief Description of the Drawings

Fig. 1 schematically shows a method for preparing a DNA fragment for deleting a gene through SOE-PCR (SOE: splicing by overlap extension) (see Gene, 77, 61 (1989), and a method for deleting a target gene (replacing the target gene with a drug resistance gene) through use of the DNA.

Modes for Carrying out the Invention

The present invention is directed to a recombinant microorganism obtained by transferring, into a host microorganism capable of producing protein or polypeptide with increased productivity, a gene encoding a protein or polypeptide, and to a method for producing a protein or polypeptide by use of the recombinant microorganism.

The present inventors have conducted extensive studies on, among many different genes encoded on the genome of a microorganism, genes which are not needed in or which are detrimental to the production of useful proteins or polypeptides, and have found that, when a gene encoding a target protein or polypeptide is transferred to a microorganism such as *Bacillus subtilis* after a specific gene is deleted or knocked out from the genome of the microorganism, productivity of the target protein or polypeptide is enhanced as compared with the case before the deletion or knocking out.

In the microorganism of the present invention, since genes which are unnecessary in or detrimental to the production or a target protein or polypeptide are deleted or knocked out, waste of culture media, including energy loss, production of byproducts, and reduced specific production rate, is significantly reduced, and in addition, protein and polypeptide can be produced over a prolonged period, whereby a target product can be produced with high efficiency.

In the present invention, homology between amino acid sequences and that between nucleic acid sequences are both determined by use of the Lipman-Pearson method (Science, 227, 1435 (1985)). Specifically, calculation is performed by use of a homology analysis program (Search Homology) developed by genetic information processing software, Genetyx-Win (Software Development Co., Ltd.), with ktup (the unit size to be compared) being set 2.

No particular limitation is imposed on a parent microorganism for constructing the microorganism of the present invention, so long as it has a gene which is not necessary for producing a target protein or polypeptide; specifically, any of the *Bacillus subtilis* genes or genes functionally equivalent thereto as shown in Table 1, wherein the gene may be of wild-type or a mutant. Specific examples include *Bacillus subtilis* and similar microorganisms belonging to the genus *Bacillus*, microorganisms belonging to the genus Clostridium, and yeast. *Inter alia*, microorganisms belonging to the genus *Bacillus* are preferred. In particular, *Bacillus subtilis* is preferred, from the viewpoint that complete genomic information of this microorganism has already been obtained, and thus genetic engineering techniques and genomic engineering techniques have been established, and that the microorganism has ability to secrete the produced protein extracellularly.

Examples of the target protein or polypeptide to be produced by use of the microorganism of the present invention

include enzymes, physiologically active substances, and other proteins and polypeptides which find utility in foods, pharmaceuticals, cosmetics, detergents, fiber-treating agents, clinical assay agents, etc.

Taking *Bacillus subtilis*, which is known to have 4,106 genes on the genome, as an example, one or more genes which are to be deleted or knocked out are any of the *Bacillus subtilis* genes shown in Table 1, or are selected from among the genes functionally equivalent thereto. The present inventors have found that such genes do not directly participate in production of the target protein or polypeptide and are unnecessary for the growth of microorganism in ordinary industrial production media.

The names, numbers, and functions of respective genes in the Tables contained herein conform with the *Bacillus subtilis* genome data reported in Nature, 390, 249-256 (1997) and made public by JAFAN (Japan Functional Analysis Network for *Bacillus subtilis*; BSORF DB) on the Internet (http://bacillus.genome.ad.jp/, renewed June 17, 2003).

Table 1

Name of the gene	Gene ID	Functions or other information of the gene
comA	BG10381	two-component response regulator
yopO	BG13648	deduced transcriptional regulator, spβ prophage protein
treR	BG11011	trehalose operon transcriptional repressor (GntR family)
yvbA	BG14078	deduced transcriptional regulator (ArsR family)
cspB	BG10824	cold shock-related major factor

yvaN	BG14069	deduced transcriptional regulator
yttP	BG13927	deduced transcriptional regulator (TetR family)
yurK	BG13997	deduced transcriptional regulator (GntR family)
yozA	BG13748	deduced transcriptional regulator (ArsR family)
licR	BG11346	transcriptional regulator (antiterminator), lichenan operon (*licBCAH*) regulation
sigL	BG10748	RNA polymerase σ factor (σ54)
mntR	BG11702	manganese transport regulator
glcT	BG12593	transcriptional regulator essential to expression of *ptsGHI* operon (BglG family, antiterminator)
yvdE	BG12414	deduced transcriptional regulator (LacI family)
ykvE	BG13310	deduced transcriptional regulator (MarR family)
slr	BG11858	transcriptional activator for competence- or sporulation-related genes
rocR	BG10723	transcriptional activator for arginine-assimilating operon (NtrC family)
ccpA	BG10376	carbon source catabolism reppression-related transcriptional regulator (LacI family)
yaaT	BG10096	type-II signal peptidase-like protein
yyaA	BG10057	DNA-binding protein SpoOJ-like protein
yycH	BG11462	Function unknown (homologous gene has been found in other organisms)
yacP	BG10158	Function unknown (homologous gene has been found in other organisms)
hprK	BG14125	Hpr protein Ser residue phosphoenzyme/dephosphoenzyme
rsiX	BG10537	anti σX factor
yhdK	BG13017	Function unknown, related to repression of σM factor expression
ylbO	BG13367	expression regulator for gene in σE-related metrocytein

Genes derived from other microorganisms, preferably from bacteria belonging to the genus *Bacillus*, which have the same functions as any of the *Bacillus subtilis* genes shown in Table 1, or have 70% or more homology with the nucleotide

sequence of any of the genes shown in Table 1, preferably 80% or more homology, more preferably 90% or more, further preferably 95% or more, yet more preferably 98% or more, should be interpreted to be functionally equivalent to the genes shown in Table 1, and thus to constitute the genes which are to be deleted or knocked out according to the present invention. In this connection, homology of nucleotides is computed by use of the Lipman-Pearson method (Science, 227, 1435, 1985).

Many of the genes shown in Table 1 which encode *Bacillus subtilis* are regulatory genes participating in activation or suppression of expression of a variety of genes, or genes deduced to be such regulatory genes. The present invention has been attained on the basis of this finding; i.e., the presence of regulatory genes unnecessary in or detrimental to production of protein or polypeptide has now been unveiled in the present invention.

Notably, attention is drawn to the fact that many of the listed "unnecessary" or "detrimental" genes are regulatory genes participating in sugar intake or metabolism, as exemplified by the *glcT* gene, which acts as an anti-terminator for a glucose PTS intake operon; the *licT* gene, which acts as an anti-terminator for a lichenan hydrolysis operon; the *treR* gene, which acts as a repressor of trehalose intake and metabolism; and the *hprK* gene and *ccpA* gene, which relate to glucose catabolite repression.

Also, in addition to the regulatory genes involved in

sugar intake and metabolism, the *rocR* gene participating in activation of arginine assimilation, and competence-related *comA* gene and *slr* gene, which are also regulatory genes, may be deleted or knocked out, to thereby improve productivity of protein or polypeptide.

The genes shown in Table 1 include the *yhdK* gene, and the *rsiX* gene encoding the anti-EC F sigma factor which suppresses expression of an ECF sigma factor, sigma x. The *yhdK* gene has been reported to participate in suppression of sigma M (Mol. Microbiol., 32, 41, 1999). The *sigL* gene, which encodes sigma L, is also included in the genes of Table 1. This suggests that expression of a gene under regulation by sigma X or sigma M is favorable for production of protein, and conversely, some gene expression under regulation by sigma L is unfavorable.

By deleting or knocking out one or more genes selected from the above-mentioned genes, expression which is unnecessary in or harmful to the production of protein or polypeptide can be prevented, leading to enhanced productivity in such production of protein or polypeptide.

The number of gene(s) to be deleted or knocked out is one or more, preferably two or more, more preferably three or more, even more preferably 5 or more. When a microorganism of the present invention is constructed, deletion or inactivation of a gene or genes other than those mentioned above is possible. In such a case, a more improved effect is expected. An alternative method for achieving the present

invention is inactivation, or knocking out, of a target gene by inserting thereto a DNA fragment of another origin or introducing a mutation to the transcription/translation-initiation region of the gene. Preferably, however, the target genes are physically deleted.

In an example procedure for deleting or knocking out the genes, any of the target genes shown in Table 1 is deleted or knocked out according to a plan which has been set up in advance. Alternatively, randomized deletion of genes or mutation by way of knocking out is performed, followed by evaluation on protein productivity and gene analysis.

The target gene may be deleted or knocked out through homologous recombination. That is, a DNA fragment containing a portion of the target gene is cloned with an appropriate plasmid vector to thereby obtain a ring-shaped recombinant plasmid, and the resultant plasmid is transferred into cells of a parent microorganism. Thereafter, through homologous recombination effected in a partial region of the target gene, the target gene on the genome of the parent microorganism is cleaved, thereby completing inactivation of the target gene. Alternatively, the target gene is knocked out by substitution or insertion of a base, or a linear DNA fragment containing a region outside the target gene sequence but not containing the target gene may be constituted through PCR or a similar method, and the thus-engineered gene or fragment is transferred into a cell of a parent microorganism. At two sites outside the mutation within the target gene in the

genome of the parent microorganism genome, or at two regions outside the target gene sequence, double crossing-over homologous recombination is caused to occur, to thereby attain substitution with a gene fragment in which the target gene on the genome is deleted or knocked out.

Particularly when the parent microorganism used to construct the microorganism of the present invention is *Bacillus subtilis*, since several reports have already described methods for deleting or knocking out the target gene (see, for example, Mol. Gen. Genet., 223, 268 1990), repetition of any of such methods may be followed, to thereby produce a host microorganism of the present invention.

Randomized gene deletion or inactivation may be performed through use of a method similar to the above-described method for inducing homologous recombination by use of a randomly cloned DNA fragment, or by way of irradiation of a parent microorganism with gamma rays or similar rays.

Next will be described in more detail a deletion method employing double crossing over by use of a DNA fragment designed for the deletion purpose, the DNA fragment being prepared through SOE-PCR (Gene, 77, 61, 1989). However, in the present invention, the method for deleting genes is not limited to only the below-described method.

The DNA fragment use for the deletion purpose is a fragment constructed such that a drug resistant marker gene is inserted between a ca. 0.5 to 3 kb upstream sequence which flanks and is upstream of the gene to be deleted, and a ca.

0.5 to 3 kb downstream sequence which flanks and is downstream of the same gene. In the first cycle of PCR, the following three fragments are prepared: the upstream and the downstream fragments, which are to be deleted, and the drug resistant marker gene. The primers to be used in this step may, for example, be those specifically designed so that an upstream 10-30 base pair sequence of a drug resistance gene is added to the lower end of the upstream fragment, and a downstream 10-30 base pair sequence of the drug resistance marker gene is added to the upper end of the downstream fragment (Fig. 1).

Next, using three PCR fragments prepared in the first cycle as templates, the second cycle of PCR is performed by use of an upper primer of the upstream fragment and a lower primer of the downstream fragment. This step causes annealing with the drug resistance marker gene fragment in the sequence of the above-engineered drug resistance marker gene, and through PCR amplification, there can be obtained a DNA fragment with the drug resistance marker gene inserted between the upstream fragment and the downstream fragment (Fig. 1).

When a chloramphenicol-resistant gene is employed as a drug resistance marker gene, a DNA fragment for deleting a gene can be obtained through SOE-PCR under typical conditions described in literature (see, for example, PCR Protocols. Current Methods and Applications, Edited by B. A. White, Humana Press, pp. 251 (1993), Gene, 77, 61, 1989), by use of

a primer set such as that shown in Table 2 and a conventional enzyme kit for PCR (e.g., Pyrobest DNA Polymerase (product of Takara Shuzo)).

When the thus-obtained DNA fragment for effecting gene deletion is introduced into cells through the competent method or a similar method, intracellular genetic recombination occurs in homologous regions which are present upstream and downstream of the gene to be deleted. Thus, cells in which the target gene has been substituted by a drug resistance gene can be selectively separated through employment of a drug resistance marker (Fig. 1). Specifically, when a DNA fragment for gene deletion prepared by use of a primer set shown in Table 2 is introduced into cells, colonies which have grown on an agar culture medium containing chloramphenicol are separated, and deletion of the target gene by way of substitution by the chloramphenicol-resistant gene is confirmed through an appropriate method such as PCR employing a genome as a template.

Subsequently, when a gene encoding a target protein or polypeptide is transferred to a host mutant microorganism strain from which any of the *Bacillus subtilis* genes shown in Table 1, or one or more genes selected from among the genes corresponding thereto has been deleted or knocked out, the microorganism of the present invention can be obtained.

No particular limitation is imposed on the gene encoding the target protein or polypeptide. Examples of the protein and polypeptide include physiologically-active

peptides and enzymes for industrial purposes such as detergents, foods, fibers, feeds, chemicals, medicine, and diagnostic agents. Industrial enzymes may be functionally grouped into oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases/synthetases. Preferably, hydrolases such as cellulase, α-amylase, and protease may be used. Specific examples include cellulase belonging to family 5 in the classification of hydrolase (Bioche M. J., 280, 309, 1991); in particular, cellulase derived from a microorganism, more particularly cellulase derived from the genus *Bacillus*. Other specific examples of the types of industrial enzymes include alkaline cellulase which is derived from the genus *Bacillus* and has an amino-acid of SEQ ID NOs: 2 or 4, and cellulase which has an another amino-acid sequence having 70% homology with said amino-acid sequence, preferably 80% homology, more preferably 90%, further preferably 95%, still further preferably 98% or more.

Specific examples of α-amylase include α-amylase derived from a microorganism, preferably liquefied amylase derived from the genus *Bacillus*. More specific examples include alkaline amylase which is derived from the genus *Bacillus* and has an amino-acid sequence of SEQ ID NO: 6, and amylase which has another amino-acid sequence having 70% homology with said amino-acid sequence, preferably 80% homology, more preferably 90%, further preferably 95%, particularly preferably 98% or more. The homology of the amino-acid sequence is calculated by the Lipman-Pearson

method (Science, 227, 1435 (1985)). Specific examples of protease include serine protease and metallo-protease which are derived from microorganisms, particularly those belonging to the genus *Bacillus*.

Preferably, a gene coding for a target protein or polypeptide has, on its upstream region thereof, one or more regulatory regions relating to transcription, translation, or secretion of the gene (specially, one or more regions selected from among a transcription initiation regulatory region including a promoter and a transcription initiation site; a translation initiation region including a ribosome-binding site and a start codon; and a secretion signal peptide region) properly ligated thereto. Preferably, it is preferred that three regions consisting of the transcription initiation regulatory region, the translation initiation regulatory region, and the secretion signal region be ligated to the target gene. Further preferably, the secretion signal peptide region is one that originates from the cellulase gene of a microorganism belonging to the genus *Bacillus*, and the transcription initiation region and the translation initiation region is a 0.6 to 1 kb region upstream of the cellulase gene. In one preferred example, a transcription initiation regulatory region, a translation initiation region, and a secretion signal peptide region of a cellulase gene derived from a microorganism belonging to the genus *Bacillus* disclosed in, for example, Japanese Patent Application Laid-Open (*kokai*) Nos. 2000-210081 and 190793/1990; i.e., a

cellulase gene derived from KSM-S237 strain (FERM BP-7875) or KSM-64 strain (FERM BP-2886), is properly ligated to a structural gene of the target protein or polypeptide. More specifically, preferred DNA fragments to be ligated include a nucleotide sequence of base numbers 1 to 659 of SEQ ID NO: 1; a nucleotide sequence of base numbers 1 to 696 of a cellulase gene of SEQ ID NO: 3; a DNA fragment having a nucleotide sequence having 70% homology with any one of said nucleotide sequences, preferably 80% homology, more preferably 90%, further preferably 95%, even more preferably 98% or more; or a DNA fragment having a nucleotide sequence lacking a portion of any one of said nucleotide sequences. Preferably, one of these DNA fragments is properly ligated to a structural gene of the target protein or polypeptide. As used herein, a DNA fragment having a nucleotide sequence lacking a portion of any one of the above-mentioned nucleotide sequences is intended to mean a DNA fragment which has functions relating to transcription, translation, and secretion of the gene, without having a portion of any one of the above-mentioned nucleotide sequences.

The recombinant microorganism of the present invention can be obtained by a conventional transformation technique in which a recombinant plasmid containing a DNA fragment which includes a gene encoding the target protein or polypeptide, and is ligated to a proper plasmid vector is transferred into a host microorganism cell. Alternatively, the recombinant microorganism may be obtained making use of a DNA fragment

prepared by ligating the above DNA fragment to a proper region which is homologous with a certain portion of the host microorganism genome, and inserted directly into a host microorganism genome.

The target protein or polypeptide obtained by use of the recombinant microorganism of the present invention may be produced in such a manner that a corresponding cell strain is inoculated onto a culture medium containing assimilable carbon sources and nitrogen sources, and other essential components; the cell strain is cultured through a conventional microorganism culturing method; and subsequently, protein or polypeptide is collected and purified.

Through the aforementioned procedure, a host mutant microorganism strain in which any of the *Bacillus subtilis* genes shown in Table 1 or one or more genes selected from genes functionally equivalent thereto have been deleted or knocked out can be engineered. In addition, by use of such a mutant strain, a recombinant microorganism can be produced. Thus, a useful protein or polypeptide can be effectively produced through employment of the mutant strain or the recombinant microorganism.

The method for constructing a recombinant microorganism according to the present invention, and the method for producing cellurase and α-amylase by use of the recombinant microorganism will next be described in detail, centering on working examples for constructing recombinant strain belonging to *Bacillus subtilis* from which the *ccpA* gene

(BG10376) of *Bacillus subtilis* has been deleted.

Examples

Example 1

A genome DNA sample, serving as a template, extracted from *Bacillus subtilis* 168 strain and two primer sets (ccpA-AF and ccpA-A/CmR; and ccpA-B/CmF and ccpA-BR) shown in Table 2 were used to prepare a 0.6 kb fragment (A) flanking the upstream side of the *ccpA* gene on the genome and a 0.6 kb fragment (B) flanking the downstream side of the *ccpA* gene. A chloramphenicol-resistant gene of plasmid pC194 (J. Bacteriol. 150 (2), 815 (1982))) was inserted into the XbaI-BamHI cleavages site of plasmid pUC18, to thereby prepare a recombinant plasmid pCBB 31. The recombinant plasmid pCBB and a primer set consisting of CmF and CmR shown in Table 2 were used to prepare a 1 kb fragment (C) containing the chloramphenicol-resistant gene. Subsequently, SOE-PCR was performed by use of the primers ccpA-AF and ccpA-BR shown in Table 2, and by use of the thus-prepared three fragments (A), (B), and (C) in combination as templates, a 2.2 kb DNA fragment in which the fragments (A), (B), and (C) were ligated in this sequence was prepared (see Fig. 1). By use of the thus-prepared DNA fragment, *Bacillus subtilis* 168 strain was transformed through the competent method. Colonies grown in an LB agar medium containing chloramphenicol were collected as transformants. The genome of the above-obtained transformant was extracted, and PCR

performed thereon confirmed that the *ccpA* gene had been deleted and substituted by a chloramphenicol-resistant gene.

Table 2-1

Primer	Nucleotide sequence	SEQ ID NO:
comA-AF	AAGGATGATAATCCGTCCCGTG	7
comA-A/CmR	GTTATCCGCTCACAATTCGGATGGTCATCAATCACTAG	8
comA-B/CmF	CGTCGTGACTGGGAAAACTGCGAAATCAGACGGTGTAC	9
comA-BR	CGTCGCCTATCGGCGGGCAC	10
yopO-AF	ATGTATATAGGAGGTTGGTGGTATG	11
yopO-A/CmR	GTTATCCGCTCACAATTCGCTCTGACATGTCAACCTCC	12
yopO-B/CmF	CGTCGTGACTGGGAAAACAGATGAGAAAGGAGGAGAAG	13
yopO-BR	ATAACTGTTACTATATAATGGCC	14
treR-AF	GCTGGGGATGACGAATCCGA	15
treR-A/CmR	GTTATCCGCTCACAATTCTCACCTTCATTATGGACCAC	16
treR-B/CmF	CGTCGTGACTGGGAAAACCACCGTCTCGACAAATTCCG	17
treR-BR	GTTGCCAAGCGCGATATAGG	18
yvbA-AF	TATACAGGGATTATCAGTATTGAGC	19
yvbA-A/CmR	GTTATCCGCTCACAATTCTTTTCTCCTTGTTGGATCTG	20
yvbA-B/CmF	CGTCGTGACTGGGAAAACGGGGATAACGATTTATGAAG	21
yvbA-BR	TTTTGTAATAATGATATGAAGCTAGTGTTG	22
cspB-AF	ATATCCAGCCCTGCCTCTTC	23
cspB-A/CmR	CTGTGTGAAATTGTTATCCGCTCACAATTCGAAATTTCCTCCTAA AGCGATCATAACG	24
cspB-B/CmF	GTCGTTTTACAACGTCGTTGACTGGGAAAACCCACAAGCTGCTAA CGTTAC	25
cspB-BR	TCCTGTTTGGGCTCCTGTTG	26
yvaN-AF	TGTTTATGTATGGCGGCCTGCGGGAC	27
yvaN-A/CmR	GTTATCCGCTCACAATTCAGCTTTCCATATATCTCACC	28
yvaN-B/CmF	CGTCGTGACTGGGAAAACACGGTCTGCTGATGACTGAC	29
yvaN-BR	GCGTTTACTTAAGATGTCGA	30
yttP-AF	TTTCTAGCGTTTCGGCAAATTGAGTTAAG	31
yttP-A/CmR	GTTATCCGCTCACAATTCCTTACTTTCATACGGCTCAC	32
yttP-B/CmF	CGTCGTGACTGGGAAAACGAGACGTGGCGCTCACCAAC	33
yttP-BR	CGGATTAAAAAAAGAATATCGCGGACAGC	34
yurK-AF	TGCCGCTGCCCGCCGGAGAG	35

Table 2-2

yurK-A/CmR	GTTATCCGCTCACAATTCAAGGTGTAGAACTTCCGTTG	36
yurK-B/CmF	CGTCGTGACTGGGAAAACACCATCAACAGCCCCTACAC	37
yurK-BR	TCAAATAAAGGCGGCATTCAGTCC	38
yozA-AF	ATAATGGTATCCAAATCCACGC	39
yozA-A/CmR	GTTATCCGCTCACAATTCATTCAGTCATATGTATCACC	40
yozA-B/CmF	CGTCGTGACTGGGAAAACGATCCATCATACACAGCATG	41
yozA-BR	CACTTCTCAACGGAGGGGATTTCACATC	42
licR-AF	TAATGGAGGAGAGAAGGCCG	43
licR-A/CmR	GTTATCCGCTCACAATTCAGTCGCCCATGAAGCATGAG	44
licR-B/CmF	CGTCGTGACTGGGAAAACACCAAAAAATGCTGAGCTGACAGC	45
licR-BR	TTGCCAATGATGAGGAAAAAGGAACC	46
sigL-AF	CTGAACGTCTTGAATAAAAAAGCAGG	47
sigL-A/CmR	GTTATCCGCTCACAATTCGCTGAAGTTTCATATCCATC	48
sigL-B/CmF	CGTCGTGACTGGGAAAACATTCCGTCATCGGCAGCGAG	49
sigL-BR	AGCGGTTTACAAGTTGGAGG	50
mntR-AF	ATTTCAGAAGGCATACTTCAAG	51
mntR-A/CmR	GTTATCCGCTCACAATTCCATACTTGGTGTTGTCATCG	52
mntR-B/CmF	CGTCGTGACTGGGAAAACCATAATCAGTAAAAAGGCGGTC	53
mntR-BR	TTCTGACCGCTCTGGCAACC	54
glcT-AF	ATAATGCCCGCTTCCCAACC	55
glcT-A/CmR	GTTATCCGCTCACAATTCCGATCCTCAGCTCCTTTGTC	56
glcT-B/CmF	CGTCGTGACTGGGAAAACTCATCTGATACCGATTAACC	57
glcT-BR	CAACTGAATCCGAAGGAATG	58
yvdE-AF	TCGGGGTCATGCCGAGCGGT	59
yvdE-A/CmR	GTTATCCGCTCACAATTCCAATGTTGCCATTTTCATCC	60
yvdE-B/CmF	CGTCGTGACTGGGAAAACTTGTACGAGAATCAACGCTG	61
yvdE-BR	CACGGCAATGCATTCTTCGG	62
ykvE-AF	AGATCTGTCGGCCAGGTTTAC	63
ykvE-A/CmR	GTTATCCGCTCACAATTCTGATTTTTCTGTCATGTCTC	64
ykvE-B/CmF	CGTCGTGACTGGGAAAACGGTAGAGATGTGCACCGAAA	65
ykvE-BR	GAGTCAGACGGCATCGATGA	66
slr-AF	TTCTGATTCATTTTCACTGCTGG	67
slr-A/CmR	GTTATCCGCTCACAATTCAACGGATAATTCTTCCAATC	68
slr-B/CmF	CGTCGTGACTGGGAAAACTGTCCATGAAGTCAAATCC	69
slr-BR	CGCTGAAATATTCTCTCGCA	70
rocR-AF	CGCCGCTTTCACCGCGGATTC	71
rocR-A/CmR	GTTATCCGCTCACAATTCCTTTGACCACTGTATGAACC	72

Table 2-3

rocR-B/CmF	CGTCGTGACTGGGAAAACACTCGTCTAACGAATAATCC	73
rocR-BR	TGTCATCACGGAATTTGACG	74
ccpA-AF	CCAAATTATCCTTTGTGAGCGCGGAATCAG	75
ccpA-A/CmR	GTTATCCGCTCACAATTCCGTAGATCGTAATATTGCTC	76
ccpA-B/CmF	CGTCGTGACTGGGAAAACAGCTTAGAAAGTCAACCAAG	77
ccpA-BR	TTTGAGCATCAGCACAAGCC	78
yaaT-AF	TGTAGCAGAAGCAGTCGAATT	79
yaaT-A/Cm2R	CTAATGGGTGCTTTAGTTGACAATTACGCAGCTGTCATGT	80
yaaT-B/Cm2F	CTGCCCCGTTAGTTGAAGAACTGATAAACCGTGAAAAAGTG	81
yaaT-RV	CCTTTGAAAAAGGCTCCCGT	82
yyaA-AF	GTTTTCCAAGTCTGCCGATAAAAATATGC	83
yyaA-A/CmR	GTTATCCGCTCACAATTCATGCTTCATGTACCTACACC	84
yyaA-B/CmF	CGTCGTGACTGGGAAAACCAATTAACGATTCGCATACC	85
yyaA-BR	AAAAAGAAGAAGTCACAGTACAGAACGTGG	86
yycH-AF	ATTTTTCGCCATCTTGAATTTTC	87
yycH-A/Cm2R	CTAATGGGTGCTTTAGTTGGATGATCCTCTCGTTGAACTG	88
yycH-B/Cm2F	CTGCCCCGTTAGTTGAAGGGATGAGCCTTCAGAAAAGTT	89
yycH-BR	GCCGGACAGAGATCTGTATG	90
yacP-B/Cm4F	GAAGAAGGTTTTTATGTTGACGCTTTTTTGCCCAATACTGTATAA	91
yacP-B/Cm4R	CAAAAAAGCGTCAACATAAAAACCTTCTTCAACTAACGGGGCAGG	92
yacP-BR	AAGACGAGTACTTTTCTCTCTAAATCACTT	93
yacP-AF	AACTCGATCAAATGGTGACAGGACAGCATC	94
yacP-A/Cm4F	GGAGAATAAAGACCCTCTTCAACTAAAGCACCCATTAGTTCAACA	95
yacP-A/Cm4R	TGCTTTAGTTGAAGAGGGTCTTTATTCTCCCACAGGGTTTCGTTT	96
hprK-B/Cm4F	TTTTTATATTACAGCGAGTTGGCGTTAAATGAATGAAGCGATAGA	97
hprK-B/Cm4R	ATTTAACGCCAACTCGCTGTAATATAAAAACCTTCTTCAACTAAC	98
hprK-BR	TTGATTGATGATAAATTCAGGCAGGTGCAG	99
hprK-AF	CAAAGCTTGAGAAATGTTCCCATGCTCTTG	100
hprK-A/Cm4F	CAGGAGGAACATATCTCTTCAACTAAAGCACCCATTAGTTCAACA	101
hprK-A/Cm4R	TGCTTTAGTTGAAGAGATATGTTCCTCCTGTTCCGGGCTGCCCCG	102
rsiX-AF	ATTCCAGTTACTCGTAATATAGTTG	103
rsiX-A/CmR	GTTATCCGCTCACAATTCACTTCATCATCCATTAGCTC	104
rsiX-B/CmF	CGTCGTGACTGGGAAAACCTGCTCCAAATCCGATTTCC	105
rsiX-BR	GTCCTGCATTTTTCGAAGTCTGG	106
yhdK-AF	TACACATCCTTCAAACAAGTCTGAACAAAC	107

Table 2-4

yhdK-A/Cm4R	TGCTTTAGTTGAAGATTACCAGTTCCATAATTCCACCTCGCCGAC	108
yhdK-B/Cm4F	TTTTTATATTACAGCGTGTGTATACCATTGTATCTGTAGATACGA	109
yhdK-BR	GCTATGATCATTGTAACGAAAGGAAAGGGG	110
yhdK-A/Cm4F	TTATGGAACTGGTAATCTTCAACTAAAGCACCCATTAGTTCAACA	111
yhdK-B/Cm4R	CAATGGTATACACACGCTGTAATATAAAAACCTTCTTCAACTAAC	112
ylbO-AF	AATCTGAACAAGAAAAAGGAGCTGCTCCTC	113
ylbO-A/Cm4R	TGCTTTAGTTGAAGAATTCAATCTCCCTCCATGTCAGCTTATTTA	114
ylbO-B/Cm4F	TTTTTATATTACAGCAGAAACGCCTGAAATGAACCGGCCCTATAG	115
ylbO-BR	TGTTTGACAAAGGTAGAACGTCTGCTTATC	116
ylbO-A/Cm4F	GGAGGGAGATTGAATTCTTCAACTAAAGCACCCATTAGTTCAACA	117
ylbO-B/Cm4R	ATTTCAGGCGTTTCTGCTGTAATATAAAAACCTTCTTCAACTAAC	118
CmF	GAATTGTGAGCGGATAAC	119
CmR	GTTTTCCCAGTCACGACG	120
Cm2F	CAACTAAAGCACCCATTAG	121
Cm2R	CTTCAACTAACGGGGCAG	122

Example 2

In a manner similar to that described in Example 1, sporulation gene-deleted strains into which a chloramphenicol-resistant gene had been introduced by way of substitution in place of the below-described deleted genes were separated through use of a DNA fragment for effecting deletion prepared from an adequate primer set selected from among various primer sets shown in Table 2; i.e., gene-AF, gene-A/CmR, gene-B/CmF, gene-BR, CmF, and CmR. The gene deleted from the genome was *comA*, *yopO*, *treR*, *yvbA*, *yvaN*, *yttP*, *yurK*, *yozA*, *licR*, *sigL*, *mntR*, *glcT*, *ykvE*, *slr*, *rocR*, *yyaA*, or *rsiX*.

Example 3

In a manner similar to that described in Example 2, a

DNA fragment for deletion was prepared by use of an adequate primer set selected from among the gene-AF, gene-A/Cm2R, gene-B/Cm2F, gene-BR, Cm2F, and Cm2R, which are shown in Table 2. By use of the thus-prepared DNA fragment, sporulation gene-deleted strains into which a chloramphenicol-resistant gene had been introduced by way of substitution in place of the below-described deleted genes were separated. The gene deleted from the genome was *cspB*, *yvdE*, *yaaT*, *yycH*, or *ylbO*.

Example 4

In a manner similar to that described in Example 2, a DNA fragment for effecting deletion was prepared from an adequate primer set selected from among the gene-AF, gene-A/Cm4R, gene-B/Cm4F, gene-BR, Cm4F, and Cm4R, which are shown in Table 2. By use of the thus-prepared DNA fragment, sporulation gene-deleted strains into which a chloramphenicol-resistant gene had been introduced by way of substitution in place of deleted genes; *yacP*, *hprK*, and *yhdK*, were separated.

Example 5

To each of the gene-deleted strains obtained in Examples 1 to 4 and to *Bacillus subtilis* 168 strain serving as a control, a recombinant plasmid pHY-S237 was introduced through the protoplast transformation method. The recombinant plasmid pHY-S237 was prepared by inserting a DNA

fragment (3.1 kb) encoding an alkaline cellulase derived from *Bacillus* sp. KSM-S237 strain (Japanese Patent Application Laid-Open (*kokai*) No. 2000-210081) into the restriction enzyme *Bam*HI cleavage site of a shuttle vector pHY300 PLK. Each of the thus-obtained cell strains was shake-cultured in an LB medium (5 mL) overnight at 30°C. The culture broth (0.03 mL) was inoculated to a 2 × L-maltose medium (2% tryptone, 1% yeast extract, 1% NaCl, 7.5% maltose, 7.5 ppm manganese sulfate 4-5 hydrate, and 15 ppm tetracycline), followed by shake culturing at 30°C for three days. After completion of culturing, cells were removed through centrifugation, and alkaline cellulase activity of the supernatant obtained from the culture was determined, thereby calculating the amount of the alkaline cellulase secreted from the cells during culturing; i.e., the amount of the extracellularly produced alkaline cellulase. As is clear from Table 3, more effective production, or secretion, of alkaline cellulase has been confirmed in the case where a gene-deleted strain was employed as a host, as compared with the control 168 strain (wild type strain).

Table 3

Name of deleted gene	Gene ID	Gene size (bp)	Size of deleted fragment (bp)	Amount of produced (secreted) alkaline cellulase (relative value)
comA	BG10381	645	588	160
yopO	BG13648	213	169	154
treR	BG11011	717	656	139
yvbA	BG14078	273	210	137
cspB	BG10824	204	171	132
yvaN	BG14069	408	379	124
yttP	BG13927	624	590	121
yurK	BG13997	729	677	118
yozA	BG13748	324	289	117
licR	BG11346	1926	1889	116
sigL	BG10748	1311	1256	114
mntR	BG11702	429	399	114
glcT	BG12593	858	811	110
yvdE	BG12414	951	916	109
ykvE	BG13310	438	356	108
slr	BG11858	459	394	105
rocR	BG10723	1386	1359	128
ccpA	BG10376	1005	957	205
yaaT	BG10096	828	828	127
yyaA	BG10057	852	816	113
yycH	BG11462	1368	1368	146
yacP	BG10158	513	513	156
hprK	BG14125	933	933	196
rsiX	BG10537	1107	1068	125
yhdK	BG13017	291	228	114
ylbO	BG13367	582	582	136
None (Wild type)	—	—	—	100

Example 6

To each of the gene-deleted strains obtained in Examples 1 to 4 and to *Bacillus subtilis* 168 strain serving as a control, recombinant plasmid pHSP-K38 was introduced through the protoplast transformation method. The recombinant plasmid pHSP-K38 was prepared by inserting, into

the restriction enzyme *Bag*II-*Xba*I cleavage site of a shuttle vector pHY300 PLK, a 2.1 kb fragment (SEQ ID No: 5) prepared by ligating an upstream 0.6 kb fragment (SEQ ID NO: 3) including portions of a promoter region and a signal sequence region of an alkaline cellulase gene with an upstream side of a DNA fragment (1.5 kb) encoding a mature enzyme region (Asp1-Gln480) of an alkaline amylase gene derived from *Bacillus* sp. KSM-K38 strain (Japanese Patent Application Laid-Open (*kokai*) No. 2000-1884882, Eur. J. Biochem., 268, 2974 (2001)). Each of the thus-obtained cell strains was shake-cultured in an LB medium (5 mL) overnight at 30°C. The culture broth (0.03 mL) was inoculated to a 2 × L-maltose medium (2% tryptone, 1% yeast extract, 1% NaCl, 7.5% maltose, 7.5 ppm manganese sulfate 4-5 hydrate, and 15 ppm tetracycline), followed by shake culturing at 30°C for three to six days. After completion of culturing, cells were removed through centrifugation, and alkaline amylase activity of the supernatant obtained from the culture was determined, thereby calculating the amount of the alkaline amylase secreted from the cells during culturing; i.e., the amount of the extracellularly produced alkaline amylase. As is clear from Table 3, more effective production, or secretion, of alkaline amylase has been confirmed in the case where a gene-deleted strain was employed as a host, as compared with the control 168 strain (wild type strain).

Table 4

Name of deleted gene	Gene ID	Gene size (bp)	Size of deleted fragment (bp)	Amount of produced (secreted) alkaline amylase (relative value)
Cultured for 3 days				
slr	BG11858	459	394	178
treR	BG11011	717	656	124
yopO	BG13648	213	169	364
yvaN	BG14069	408	379	148
yvbA	BG14078	273	210	171
None (Wild type)	—	—	—	100
Culture for 5 days (Wild type)				
cspB	BG10824	204	171	195
rocR	BG10723	1386	1359	215
sigL	BG10748	1311	1256	204
glcT	BG12593	858	811	132
yvdE	BG12414	951	916	127
yacP	BG10158	513	513	110
None (Wild type)	—	—	—	100
Cultured for 6days				
yycH	BG11462	1368	1368	120
licR	BG11346	1926	1889	122
None (Wild type)	—	—	—	100